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TITLE: New Diagnostic and Therapeutic Approaches to Eradicating Recurrent Breast Cancer

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CONTRACTING ORGANIZATION: Brigham and Women's Hospital
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THE BRIGHAM AND WOMEN'S
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14. ABSTRACT

Some breast cancer patients have no evidence of metastatic disease when they are first diagnosed, yet many patients later return to the clinic with cancer that has spread throughout the body. It is thought less than 1% of the cells that disseminate are able to form overt tumors. The reasons why certain disseminated tumor cells remain inconsequential and others form life-threatening tumors after long periods of time are unknown. Support from the Era of Hope Scholar Award enabled us to use a sensitive new technology to tag individual tumor cells, each with its own unique label, and trace the individual cells in our breast cancer metastasis models. Our new detection methods enabled us for the first time to isolate and study the consequential cells (those that formed tumors) and likewise, determine which tumor cells did not form a tumor. By distinguishing consequential from inconsequential breast cancer cells, we hope to provide a foundation for future work to determine whether the disseminated tumor cells isolated from breast cancer patients have similar features. Success in these endeavors would mean that breast cancer patients harboring potentially life-threatening disseminated tumor cells could be identified and treated *before* they experience disease relapse.

15. SUBJECT TERMS
Breast cancer, metastasis, dissemination, recurrence, therapeutic resistance, systemic instigation, microenvironment, bone marrow cells, canine, mouse models

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Our ultimate goal in conducting this Era of Hope Scholar Award study is to develop new non-invasive tests that will allow oncologists to more accurately identify breast cancer patients who are likely to suffer from disease relapse and to identify new treatment therapies that can be given to those patients *before* disease recurs. **We hypothesize that certain disseminated tumors are endowed with properties that enable them to respond to specific systemic and microenvironmental cues to become malignant metastases and that neutralizing these tumor-promoting processes will provide a therapeutic strategy to save lives**. To rigorously test this hypothesis, we proposed the following:
Aim 1: Define a set of DTC biomarkers that predict risk of breast cancer recurrence
Aim 2: Develop a low-cost, non-invasive test for breast cancer recurrence risk stratification
Aim 3: Identify existing drugs that prevent malignant conversion of DTCs
Our studies are being performed using breast cancer cells, mouse models of breast cancer, and breast cancer patient blood samples and tumor tissues in order to test these new strategies before trying them in patients. We are using a new, highly innovative and sensitive technology that enables us to study rare events related to metastatic outgrowth in vivo, which was previously impossible to do. Our studies are designed to provide us with the first precise identity of life-threatening human cancer cells before they convert to a malignant state. We are also using a unique co-culture assay, developed in our laboratory, to identify mechanisms by which indolent cells convert to malignancy and to identify existing drugs that can prevent their conversion. For this project, we have brought together a team of clinical oncologists, breast pathologists, patient/research advocates, computational biologists, and veterinary oncologists in order to leverage opportunities for immediate clinical translation of our research findings.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Breast cancer, metastasis, dissemination, recurrence, therapeutic resistance, systemic instigation, microenvironment, bone marrow cells, canine, mouse models

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the USAMRAA Grants Officer whenever there are significant changes in the project or its direction.

- What were the major goals and objectives of the project?
- What was accomplished under these goals?
- What opportunities for training and professional development did the project provide?
- How were the results disseminated to communities of interest?
- What do you plan to do during the next reporting period to accomplish the goals and objectives?

A. What were the major goals of the project?

Task 1: Define tumor cell hallmarks that predict risk of breast cancer recurrence

a. Identify human breast cancer barcoded DTCs that convert to malignancy in xenograft mouse models of metastasis – **100% complete in one model; 40% complete for bone metastasis model**
b. Identify mouse Her2+ barcoded DTCs that convert to malignancy in model of metastasis – **50% complete**
c. Test select human and mouse barcoded DTCs individually *in vivo* – **75% complete**
d. Define molecular profile of mouse and human barcoded DTCs via L1000 technology – **60% complete**
e. Characterize mouse and human barcoded DTCs *in vitro* – **80% complete**
f. Analyze data and build prediction signatures (months 12-24) – **10% complete**
g. Establish predictive power of molecular/cellular signatures using other cell lines and human tumor specimens (months 12-48) – **not initiated**
h. Meet with project team to discuss findings and potential for clinical translation (months 6, 18, 30) – **100% complete for this funding period**

Task 2: Develop a low-cost, non-invasive test for breast cancer recurrence risk stratification

a. Determine ability of various human and mouse BMDC populations to induce malignant conversion of a test set of DTCs *in vitro* (months 1-36) – **30% complete**
b. Validate findings from "a" *in vivo* (months 24-48) – **not initiated**
c. Validate findings on TMAs constructed from large cohort of breast cancer patients (months 24-36) – **not initiated**
d. Determine response of various barcoded DTCs from human and mouse and human DTC samples to pro-tumorigenic BMDCs *in vitro* (months 1-36) – **25% complete**
e. Validate findings from "d" *in vivo* (months 24-48) – **not initiated**
f. Meet with project team to discuss findings and potential for clinical translation (months 6, 18, 30, 42) – **100% complete for this funding period**

Task 3: Identify existing drugs that prevent malignant conversion of otherwise indolent tumors

a. Determine ability of various drugs/compounds to prevent malignant conversion of human and mouse DTCs *in vitro* (months 24-60, approx. 12 compounds/year) – **not initiated**
b. Validate select drugs from "a" *in vivo* (months 24-60) – **not initiated**
c. Perform proteomic analyses and ELISAs on candidate tumor-promoting factors (months 36-60) – **not initiated**
d. Determine ability of various BMDCs to confer resistance to anti-cancer drugs *in vitro* (months 24-60) – **not initiated**
e. Validate select findings from "d" *in vivo* (months 24-60) – **not initiated**
f. Perform proteomic analyses and ELISAs on candidate tumor-resistance factors (months 36-60) – **not initiated**
g. Meet with project team to discuss findings and potential for clinical translation (months 18, 30, 42, 54) – **not initiated**

B. What was accomplished under these goals?

The majority of our efforts in this first year of funding have been focused on our first aim to define tumor cell hallmarks that predict risk of breast cancer recurrence. To do so, we are using mouse models of breast cancer progression during the early phases of metastatic disease when patients harbor indolent disseminated tumor cells in the periphery at the time of their primary diagnosis. We generated a population of uniquely labeled human breast tumor cell clones that represent DTCs from patients with TNBC. This collection of cell clones is ideal for distinguishing consequential from inconsequential DTCs and provides us with a powerful and innovative tool for our proposed studies of the fundamental molecular events responsible for incurable breast cancer. We tagged each individual clonal population with a unique molecular "barcode" sequence using lentiviral vectors, developed by our colleagues at the Broad Institute. Upon integration, each vector introduces a unique heritable barcode tag into each cell clone genome; hence, we can precisely follow the progeny of each cell over time. Each variable barcode sequence is flanked by uniform sequences, common to all barcode vectors, which allows for PCR amplification of barcodes from genomic DNA. To identify and quantify relative abundance of each clonal population within a polyclonal mixture of cells, including tumor and non-tumor stromal cells, we incubate PCR products with Luminex beads linked to antisense oligonucleotides corresponding to each unique barcode. We are thereby able to identify and quantify the representation of each individual barcode via Luminex FlexMap detector. Using these clones, we conducted the following experiments:

1. Using our model of triple-negative breast cancer (TNBC), we injected a pool of 30 barcoded TNBC clones to determine which disseminated tumor cells (DTCs) respond to systemic stimuli to form overt tumors and those that remain indolent, or are not detected in the resultant tumors. We have completed 3 independent experiments that yielded consistent results. Preliminary assessment of these results revealed that intratumoral sub-clonal diversity was significantly enhanced ~2.7-fold in animals with TNBC relative to the control cohort. In the absence of a tumor-supportive environment, tumors formed with an average of 4.5 clones per tumor, whereby 6 different clones were represented across the entire cohort and 4 specific clones emerged in all tumors. In this control cohort, the level of clonal diversity was maintained regardless of tumor mass, which ranged from 250 mg to 890 mg. In contrast, the tumors that formed from DTCs in mice with a primary TNBC were comprised of an average of 12 clones per tumor and 14 different clones were represented among the entire cohort. The same clones that were common among all tumors from the control cohort were also represented in all tumors from the TNBC cohort, irrespective of tumor size, which ranged from 65 mg to 332 mg. For the most part, the same clones were detected in all regions of a given tumor; however, in the largest tumor from the TNBC cohort, some clones

only emerged in specific tumor regions. (**See Figs. 1 and 2**).

2. We performed in vitro characterization of each barcoded clonal population of TNBC cells for proliferation kinetics, tumorsphere forming ability, and expression of various cell surface markers (**See Figs. 3 and 4**).

3. Based on our surprising in vivo results, we created the following new sub-pools of barcoded TNBC clones:

Pool A	The 6 clones that formed tumors with low incidence without systemic stimulation
Pool A'	A pool of 6 clones that did not appear in any tumors, as a size-matched control to Pool A
Pool C	The 14 clones that formed tumors in response to systemic stimuli provided by primary TNBC
Pool C'	A pool of 14 clones that did not appear in any tumors, as a size-matched control to Pool C
Pool D	A pool of 9 clones that appeared exclusively in tumors that responded to systemic stimuli (i.e. pool C – pool A = pool D)
Pool D'	A pool of 9 clones that did not appear in any tumors, as a size-matched control to Pool D
Pool E	The 15 clones that were inconsequential and did not appear in tumors under any circumstances

4. We performed gene expression analysis (L1000) of each individual clonal population in vitro, in both full cell culture medium as well as growth factor-deprived medium. The L1000 method is based on analysis of 1,000 carefully-selected transcripts from which the remainder of the transcriptome can be computationally inferred. This approach utilizes Luminex beads and provides a high throughput (384-well based) method. Interestingly, clones that formed tumors under various conditions did not cluster, indicating that the resulting tumors retain heterogeneity with respect to tumor cell phenotype. We next performed gene set enrichment analyses (GSEA) on the various pools of clones (ie., that were created based on in vivo results). These analyses revealed that the tumor cells that responded to the systemic environment established by a primary TNBC (pools C and D) expressed sets of genes involved in: epithelial-mesenchymal transition, inflammation, hypoxia, and xenobiotic metabolism, relative to DTCs that formed without systemic stimuli (pool A) or those that were inconsequential (pool E). These analyses were performed on the cells prior to them forming a tumor. (**See Table 1**).

5. We established additional metastasis models – lung and bone - in which the barcoded clones can be tested in the next year. We have initiated experiments to injected barcoded clones intraveneously through the tail vein (lung metastasis) and intracardiac and inra-tibial injections for bone metastases. (**See Fig. 5**)

6. As related to Aim 2 of our project, we have started to test the effects of common cancer therapeutics on the clonal composition and resistance/sensitivity of our barcoded pools of cells. We found that the barcoded TNBC clones that form tumors in the mice with TNBC are less sensitive to the cytotoxic effects of 5FU than other clones that remain indolent or that did not emerge in vivo. (**See Fig. 6**).

7. As related to Aim 2, we have been working to optimize our in vitro protocols so that over the coming years, we can test various bone marrow cells and human cells for their pro-tumorigenic function

8. We held a project retreat that included members of the lab, one of our clinical collaborators (Dr. Andrea Richardson), or scientific colleague (Dr. Christine Chaffer), and 2 breast cancer research/patient advocates (Liz Frank and Ruth Fax. The retreat was held over a 2-day period in which investigators gave formal presentations of their work in progress and we had dedicated discussion time for feedback and exchange of ideas.

C. What opportunities for training and professional development has the project provided?

Funds from this project enabled postdoctoral associates and the graduate student to attend one international scientific conference, where they gave poster or oral presentations. For example, students attended the San Antonio Breast Cancer Conference.

We were also able to hire 2 undergraduate summer interns, who each worked on aspects of the project under my supervision as well as that of postdoctoral fellows. This opportunity not only allowed summer interns to gain valuable training, but also provided the postdocs with important mentorship opportunities.

D. How were the results disseminated to communities of interest?

During the past year, we had a number of opportunities to interact with the Dana Farber/Harvard Cancer Center breast cancer research and patient advocates. I was invited to present our work to the group during their monthly meeting. Two of the advocates (Liz Frank, Lead Advocate and Ruth Fax, Advocate) attended our retreat where they each gave a presentation to the group. Liz presented an overview of the goals and operations of the advocate group and how they can help us translate our research findings to the clinic. Ruth gave a presentation on effective grant writing and what DOD review committees look for.

E. What do you plan to do during the next reporting period to accomplish the goals?

Related to Aim 1: In the next year, we will use the barcoded pools to answer a number of questions about the consequences of enhanced heterogeneity observed in the tumors that responded to systemic stimuli. For example, we will test whether sensitivity to various chemotherapeutic drugs is different among them. We plan to expand our findings into other models of breast cancer metastasis, including bone metastasis models and immunocompetent mouse models. We will continue to perform computational analyses of the data in order to build prediction signatures and establish the predictive power of the molecular and cellular signatures that we generate. We hope to submit a paper for publication on this topic.

Related to Aim 2: As we have gathered experimental tools and optimized our protocols, we will begin to assess the ability of various human and mouse bone marrow derived cell populations to induce malignant conversion of a test set of DTCs *in vitro*. We also hope to validate our findings in vivo using our mouse models as well as tissue specimens from breast cancer patients.

We will continue to hold regular meetings with the project team to discuss findings and assess the potential for clinical translation.

4. IMPACT: This component is used to describe ways in which the work, findings, and specific products of the project have had an impact during this reporting period. Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

- the development of the principal discipline(s) of the project;
- other disciplines;
- technology transfer; or
- society beyond science and technology.

What was the impact on the development of the principal discipline(s) of the project?

Our proposed lines of investigation will provide us, and the wider cancer research community, with novel tools and a highly useful collection of breast cancer DTCs that can be used to answer important and highly clinically relevant questions. We propose to test not only cell lines, but cells derived from primary breast cancer patient tumor specimens in a similar fashion. Therefore, our proposal incorporates immediate translation to human breast cancer patients. Importantly, our proposed studies should pave the way, in the very near future, toward identifying whether cancer patients harbor harmful DTCs in their circulation or bone marrow. Indeed, it is becoming common practice in the field to isolate circulating and disseminated tumor cells from cancer patient blood and bone marrow samples, both in the U.S. and in Europe; however, it is currently impossible to know the relevance of these cells to disease recurrence. As such, our studies serve as a necessary prerequisite for more accurate identification of breast cancer patients who would benefit from adjuvant therapy.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

- Changes in approach and reasons for change.
- Actual or anticipated problems or delays and actions or plans to resolve them.
- Changes that have a significant impact on expenditures.
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

We will have a carry forward in personnel support because this fund was allocated to support 2 postdoc's and we are currently support just one of those slots with a portion of Jaclyn and Zafira. In addition Amey Barakat left early and Anna Molineaux who replaced her just started so the support for a research technician/lab manager was not completely expended in year 1. In regards to the travel, the collaborative meeting which included Elizabeth Frank our patient advocate was scheduled late in the budget year. Thus the travel expenses for this meeting will not be reflected until the first month of the 2nd budget year.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

6. PRODUCTS: List any products resulting from the project during the reporting period. Examples of products include:

- publications, conference papers, and presentations;
- website(s) or other Internet site(s);
- technologies or techniques;
- inventions, patent applications, and/or licenses; and
- other products.

If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Nothing to report

- **Technologies or techniques**
  Nothing to report

- **Inventions, patent applications, and/or licenses**
  Nothing to report

- **Other Products**
  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Provide the following information on participants:

- what individuals have worked on the project?
- has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period?
- what other organizations have been involved as partners?

What individuals have worked on the project?

Name:	Sandy McAllister
Role:	Principal Investigator
Nearest person month worked:	6
Contribution to project:	Dr. McAllister oversees all aspects of the project and supervises personnel on the project
Name:	Tyler Laszewski
Role:	Research Technician
Nearest person month worked:	9
Contribution to project:	Mr. Laszewski performs and supports all animal work associated with the project
Name:	Amey Barakat
Role:	Research Technician/Lab Manager
Nearest person month worked:	9
Contribution to project:	Ms. Barakat provided technical support to the project
Name:	Jessica Hawkins
Role:	Graduate Student
Nearest person month worked:	10
Contribution to project:	Ms. Hawkins has performed work in the area of tumor cell barcoding and metastasis
Name:	Jaclyn Sceaney
Role:	Postdoctoral Fellow
Nearest person month worked:	4
Contribution to project:	Dr. Sceneay has performed work in the area of bone marrow cell analysis
Name:	Anna Molineaux
Role:	Research Scientist/Lab Manager
Nearest person month worked:	1
Contribution to project:	Ms. Molineaux provides technical support to the project
Name:	Virginia Bruch
Role:	Graduate summer student
Nearest person month worked:	3
Contribution to project:	Ms. Bruch has performed work on defining molecular properties of metastatic tumor cells
Name:	Francis Greathouse
Role:	Undergraduate summer student
Nearest person month worked:	3
Contribution to project:	Ms. Greathouse performed work in the area of molecular barcoding of tumor cells

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes.

Since the activation of this award the following grants have ended for Dr. McAllister:

Elucidating the Pathophysiology of Disseminated Tumor Cells
Gertrude B. Elion Cancer Research Award (McAllister, PI) 07/1/13-6/30/14 1.80 mos/yr
American Association for Cancer Research
Margaret Foti, M.D., Ph.D., Executive Officer
615 Chestnut Street, 17th Floor
Philidelphia, PA 19106
The goal of this project is to understand how certain indolent tumors respond to systemic factors to become overtly proliferating tumors. We aim to characterize the phenotype and molecular profile of these tumors.
THERE IS NO OVERLAP WITH THE ERA OF HOPE SCHOLAR AWARD.

Elucidating the Pathophysiology of Disseminated Breast Tumor Cells
2014 Exceptional Project Grant (McAllister, PI) 01/01/14-12/31/14 1.20 mos/yr
Breast Cancer Alliance
Kathy Hanson, RN, MBA
Karen Lowney, PhD, JD
Research Grants Co-Chairs
The Breast Cancer Alliance
48 Maple Avenue
Greenwich, CT 06830
203-861-0014
researchgrants@breastcanceralliance.org
This project is designed to identify the molecular basis by which bone metastases form using a preclinical model of breast cancer metastasis.
THERE IS NO OVERLAP WITH THE ERA OF HOPE SCHOLAR AWARD

The following award was activated:

Understanding How the Aging Hematopoietic System Affects Cancer Progression
R21 Exploratory Development Grant (TRACI) 09/01/13-08/31/15 1.20 mos/yr
NIH/NCI (McAllister, PI)
The aims of this project are to understand how age affects tumor-supportive hematopoietic cells and in turn, how these processes ultimately impact breast cancer progression using two different mouse models.

What other organizations were involved as partners?

Organization Name: Broad Institute of Harvard and MIT

Location of Organization: (if foreign location list country) Cambridge, Massachussetts

Partner's contribution to the project (identify one or more) Collaboration

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES: None

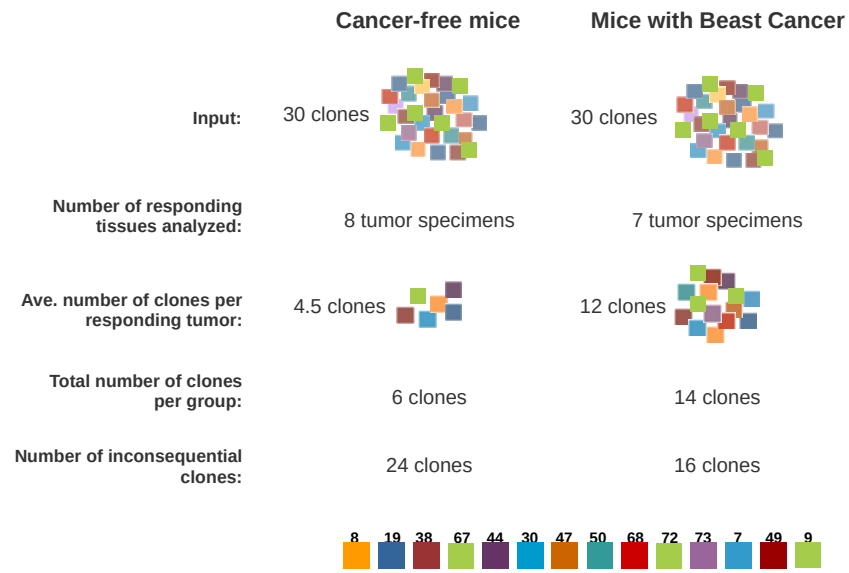

Figure 1. Results from in vivo experiments to identify TNBC clones that form a tumor under control conditions (cancer-free mice, left) or following systemic stimuli provided by a primary breast cancer at a distant anatomical site (mice with breast cancer, right). Clones that are inconsequential were also identified using these approaches.

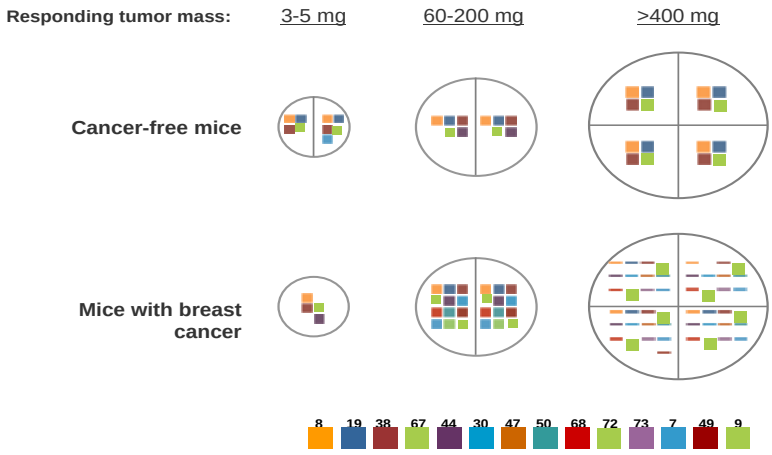

Figure 2. Comparison of clonal heterogeneity and representation of clonal breast cancer cells among size-matched tumors; representative tumors drawn as circles. Quadrants indicate special representation of various clones within any given tumor.

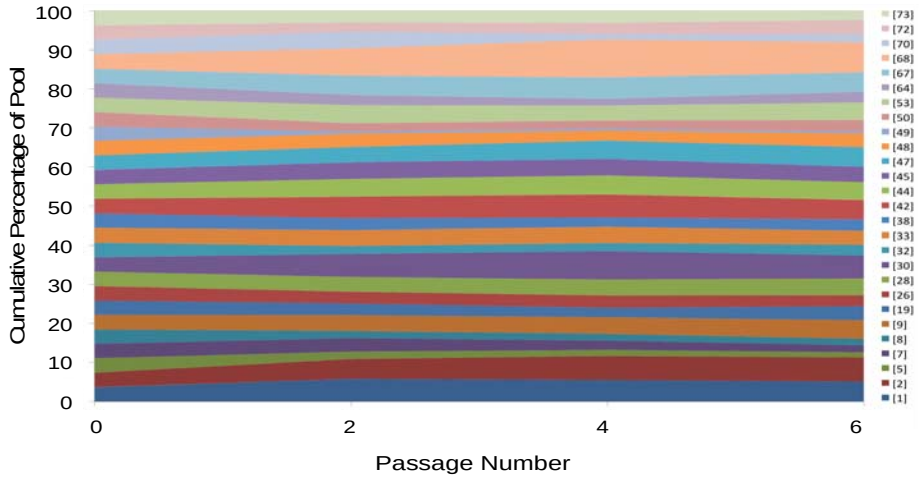

Figure 3. Representation of each barcoded clonal population within the heterogeneous mixture of cells with each passage in culture. . Each color represents a different clone. At the beginning of the experiment ("passage 0") each clone represents 3.3% of the total population.

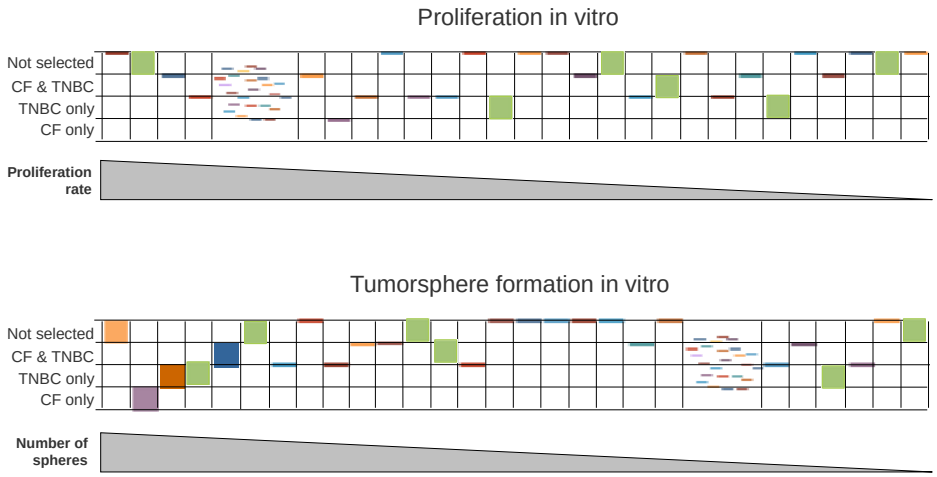

Figure 4. Analysis of proliferation kinetics and tumor sphere formation of each individual clonal population of cancer cells used for this project. Each color represents a different clone. Clones are ranked in order of highest to lowest proliferation rate (top) or ability to form tumor spheres (bottom). Rows indicate biological behavior in vivo with respect to indolence or tumor formation under indicated conditions.

Positive Enrichment

gene_set	A	B	C	D	E	F	total
HALLMARK_XENOBIOTIC_METABOLISM_DN	0	0	0	0	1	0	1
HALLMARK_NOTCH_PATHWAY	1	1	0	0	1	0	3
HALLMARK_INTREFERON_RESPONSE_UP	0	1	0	0	1	0	2
HALLMARK_INFLAMMATION_UP	1	1	0	0	0	1	3
HALLMARK_IL6_JAK_STAT3_PATHWAY	0	0	0	1	0	1	2
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION_DN	0	0	0	0	1	0	1
HALLMARK_ANDROGEN_RESPONSE	1	0	0	0	0	0	1

Negative Enrichment

gene_set	A	B	C	D	E	F	total
HALLMARK_XENOBIOTIC_METABOLISM_DN	0	0	1	1	0	0	2
HALLMARK_SHH_PATHWAY	0	0	1	1	0	0	2
HALLMARK_OXPHOS_DN	0	0	1	1	0	0	2
HALLMARK_NOTCH_PATHWAY	0	0	1	1	0	0	2
HALLMARK_INTREFERON_RESPONSE_DN	0	0	1	1	0	0	2
HALLMARK_INFLAMMATION_DN	0	0	1	1	0	0	2
HALLMARK_HYPOXIA	0	0	1	1	0	0	2
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION_DN	0	0	1	1	0	0	2
HALLMARK_DNA_DAMAGE_RESPONSE_DN	0	0	1	1	0	0	2
HALLMARK_COMPLEMENT_DN	0	0	1	1	0	0	2
HALLMARK_APICAL_JUNCTION	0	0	1	1	0	0	2
HALLMARK_ANDROGEN_RESPONSE	0	0	0	1	0	0	1
HALLMARK_APOPTOSIS	0	0	0	1	0	0	1

Table 1. Gene set enrichment analysis of indicated pools (A through E) of barcoded breast cancer cells. Positive enrichment indicates sets of genes in defined pathways with which any given pool is significantly positively correlated. Negative enrichment indicates sets of genes in defined pathways with which any given pool significantly inversely correlates. Pools are scored in a binary fashion, where 1 indicates correlation and 0 indicates no correlation.

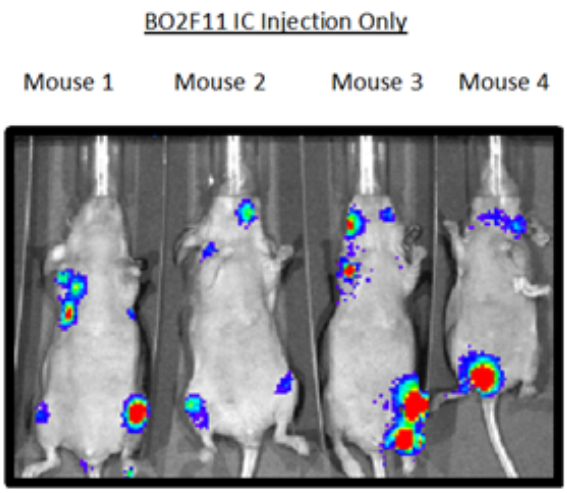

Figure 5. Human triple negative BO2F11 cells labeled with luciferase were injected intracardiac (IC) to form bone metastases that are readily observable via bioluminescence detection

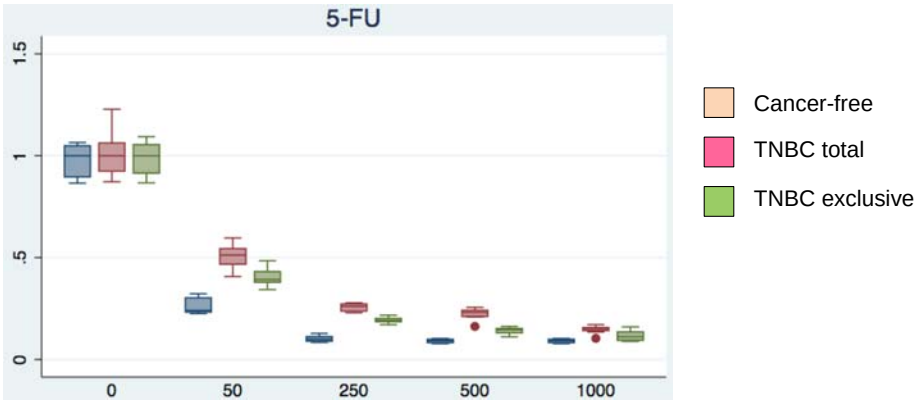

Figure 6. Response of indicated TNBC barcoded pooled populations in response to 5-FU in vitro.